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Raffaelea quercus-mongolicae sp. nov. associated with *Platypus koryoensis* on oak in Korea

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Abstract — A previously undescribed fungus frequently isolated in Korea from dead oak trees (e.g., *Quercus mongolica, Q. aliena, Q. serrata*) attacked by the ambrosia beetle, *Platypus koryoensis,* is described as *Raffaelea quercus-mongolicae.* Phylogenetic analysis of 18S rDNA sequences shows the new species to be closely related to *R. quercivora*, a causal agent of oak mortality in Japan, while ITS rDNA and β-tubulin sequence analyses reveal significant differences. *Raffaelea quercus-mongolicae* also differs from *R. quercivora* in conidial shape, associated ambrosia beetle species, geographic origin, and host range.

Key words — *Ambrosiella*, morphology, *Ophiostomatales*, phylogeny, symbiont

Introduction

Since 2004, an unknown fungus has been isolated, most frequently from dead mongolian oak (*Quercus mongolica*), but also (rarely) from *Q. aliena* and *Q. serrata* in the central part of Korea. Kim et al. (2005, 2008) note that the epidemic continues and is spreading southwards. The causal agent is believed to be closely associated with a wood boring ambrosia beetle, *Platypus koryoensis* (*Coleoptera: Curculionidae: Platypodidae*) in that both fungus and beetle were simultaneously observed from dead or infected oak, and the fungus was also isolated from the beetles (Kim et al. 2005). Male and female beetles, both of which have mycangial cavities adapted for carrying a symbiont fungus, make galleries that serve as entry points for the fungus and which lead directly into the sapwood of the tree (Moon et al. 2008).

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As with most ambrosia beetle-associated symbionts (Batra 1967), the unknown isolates formed sporodochia in beetle galleries that produced conidiophores and conidia morphologically similar to those produced by representatives of the genera, *Raffaelea* and *Ambrosiella* (*Ophiostomatales*). However, it is notoriously difficult to differentiate these two *Ophiostoma*-associated anamorphic genera solely through morphology (Harrington 2005). Previous phylogenetic analysis (Gebhardt et al. 2005) showed that *Raffaelea*-based 18S rDNA sequences are scattered throughout *Ambrosiella.* Thus, Harrington et al. (2008) proposed that *Raffaelea* is the most appropriate name for all ambrosia beetle symbionts with affinities to *Ophiostoma* until the taxonomy of the large genus *Ophiostoma* is resolved. Sequence analysis of the 18S rDNA region showed the Korean isolates embedded within *Raffaelea.* Of the twelve species thus far described in the genus *Raffaelea* (Jones & Blackwell 1998, Kubono & Ito 2002, Harrington et al. 2008), the Japanese fungus *R. quercivora* shares several characters with the present Korean isolates but differs in the associated ambrosia beetle and host plant species and conidial morphology. Although the differences would suggest that the Korean isolates might represent a new *Raffaelea* species, more extensive study was considered necessary before introducing a new taxon.

This paper documents the phenetic characters of the Korean isolates, as well as sequence analyses of the partial 18S rDNA, the completed ITS rDNA, and the partial β-tubulin regions of three selected isolates in comparison with other *Raffaelea* species. In particular, the Korean species is compared with *R. quercivora*, a causal agent of oak mortality in Japan. A new taxon is described and illustrated as *R. quercus-mongolicae*.

Materials and methods

Fungal isolates — About one hundred isolates of *Raffaelea* sp. were collected from infected or dead *Quercus mongolica*, *Q. aliena*, and *Q. serrata* trees or from mycangia of *Platypus koryoensis*. Three representative isolates, which are maintained in the Korean Agricultural Culture Collection, Suwon, Korea (KACC), were morphologically and molecularly analyzed in this study. For comparison, two cultures of *R. quercivora* (MAFF410918, MAFF12457) and one of *R. canadensis* (CBS 168.66) were obtained from the National Institute of Agrobiological Resources, Tsukuba, Japan (MAFF) and the Centraalbureau voor Schimmelcultures, the Netherlands (CBS), respectively.

Morphological analysis — Microscope slide preparations were examined in bright field and DIC light microscopy, using an Olympus BX51 microscope (Olympus, Tokyo, Japan) for measurements and a Zeiss AX10 microscope (Carl Zeiss, Göttingen, Germany) mainly for photography. Measurements were taken at 1000× for conidia and at 100–200× for other organs. They are reported as maxima and minima in parentheses, and the mean plus and minus the standard deviation. Means are shown in italic in the centre of the measurements. The surface structure of conidia was observed and

photographed with a Hitachi S-3500N scanning electron microscope. For SEM, the conidiophores and conidia were attached to holders by double-sided adhesive tape and coated with platinum with a Hitachi E-1010 Ion Sputter.

Phylogenetic analysis — G-DNA was extracted from conidiophores and conidia grown on PDA plates, in accordance with Lee & Taylor (1990). For 18S rDNA amplification and sequencing, primers NS1 and NS6 (White et al. 1990) were employed; ITS1-F (Gardens & Bruns 1993) and ITS4 (White et al. 1990) were used for ITS rDNA regions; T10 and BP12 (Kim et al. 2003) were used for β-tubulin regions. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced on an automatic sequencer (ABI Prism TM 377 DNA Sequencer), using BigDye™ (Applied Biosystems, Foster City, CA, USA) Cycle Sequencing Kit, version 3.1, with the same primers as used for amplification of the three regions.

The newly obtained sequences were edited using DNASTAR computer package (Lasergene, Madison, WI), version 5.05. Sequences were aligned using CLUSTAL X (Thompson et al. 1997). Phylogenetic trees were generated using maximum likelihood (ML) and maximum parsimony (MP) methods. For ML inference, RAxML (Stamatakis 2006) version 7.0.3 was used with all parameters set to default values, using the GTRCAT variant. A MP heuristic search was performed with 1000 random sequence additions and branch swapping by tree bisection-reconnection, using PAUP* version 4b10 (Swofford 2002). For both analyses, the relative robustness of the individual branches was estimated by bootstrapping using 1000 replicates.

Results

Phylogenetic analysis

About 1450 bp of a partial 18S rDNA were amplified and sequenced from each isolate. The Korean isolates and *R. quercivora* showed no large insertion, which had appeared previously in some *Raffaelea* species (Jones & Blackwell 1998). The beginning of ITS1 and end of ITS2, which are significantly conserved, were determined and adjusted by comparing with other *Ophiostomatales* sequences. The phylogenetic relationships between *Raffaelea* isolates were inferred from ML and MP analyses of three data sets of the partial 18S, the complete ITS rDNA, and the partial β-tubulin gene. The results of the phylogenetic reconstructions by ML inference are shown in Figs. 1-A (18S rDNA), 1-B (ITS rDNA), and 1-C (β-tubulin gene).

In the 18S rDNA alignment, 45 of the 1271 characters were parsimony-informative, and the MP analysis resulted in a most parsimonious tree of 129 steps, with a CI and RI of 0.8295 and 0.8181, respectively. For ITS rDNA alignment, 37 of the 558 characters were parsimony-informative, and the parsimony analysis produced a most parsimonious tree of 126 steps. For β-tubulin gene alignment, 62 of the 881 characters were parsimony-informative, and the parsimony analysis produced a most parsimonious tree of 153 steps. In the latter two trees, both CI and RI were 1.0000. Since no differences were

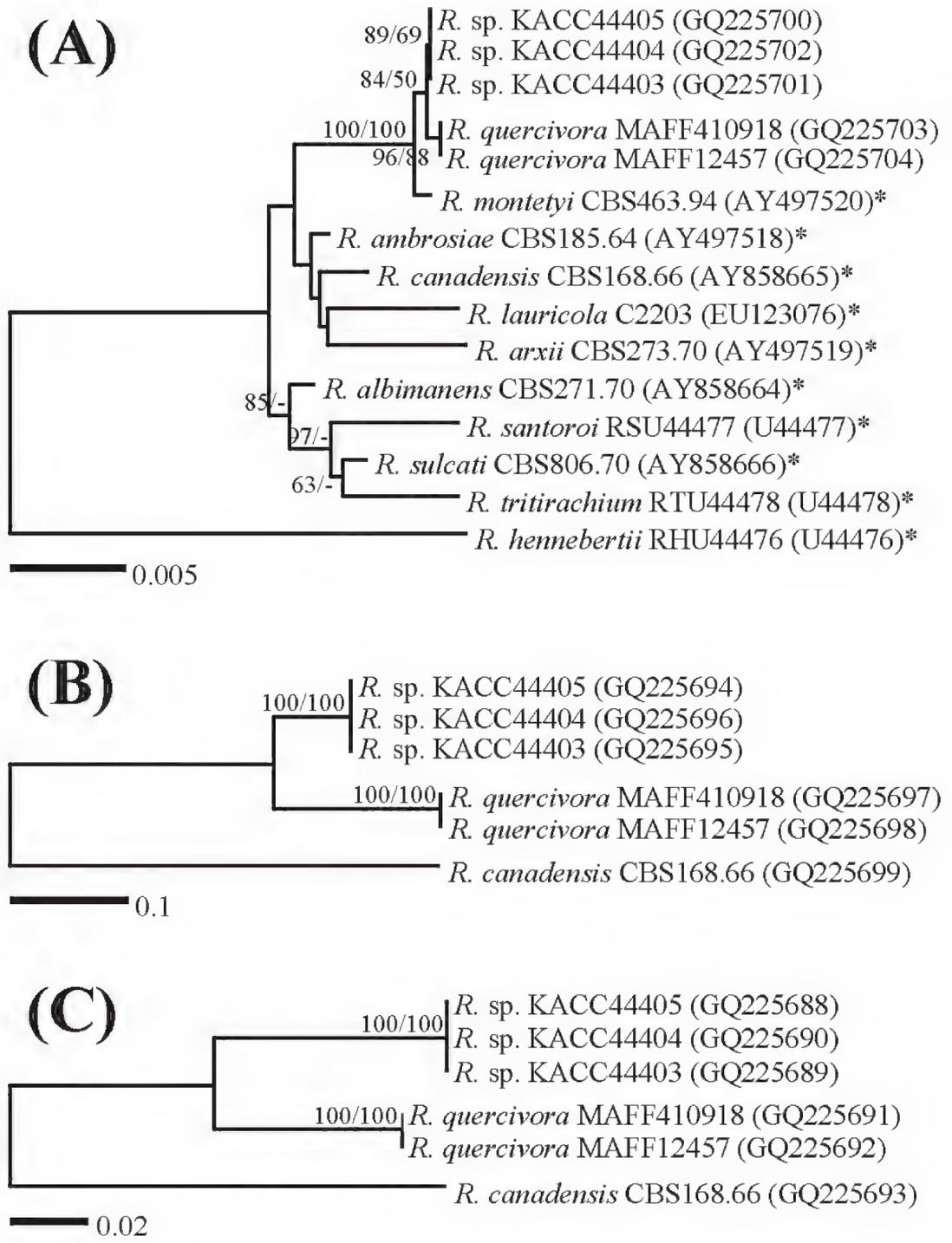

FIGURE 1. Phylogenetic trees of *Raffaelea* species inferred by ML analysis using (A) the partial 18S rDNA, (B) the complete ITS rDNA, and (C) the partial β-tubulin gene. ML and MP bootstrap values above 50 % are given above the branches. The number of nucleotide changes between taxa is represented by branch length and the scale bar equals the number of nucleotide substitutions per site. An asterisk (*) indicates the sequence obtained from GenBank.

found between the ML and MP tree topologies, only the ML trees (with MP analysis support values added) are shown.

The phylogenetic relationships between the Korean isolates and other *Raffaelea* species can only be evaluated from the 18S tree, as neither ITS nor β-tubulin sequence of other *Raffaelea* species were available from GenBank. In the 18S tree, *Raffaelea* sp. (from *Q. mongolica*), *R. quercivora*, and *R. montetyi* formed a group with a high supporting value of 100% in ML and MP analyses, distantly related to other *Raffaelea* species. The first two species were further clustered to a subgroup with moderate ML and MP bootstrap values of 84 % and 50 %, respectively. The phylogenetic distances of "*R.* sp." to *R. quercivora* were slight; they differed only at two positions out of 1271 nucleotide characters, whereas "*R.* sp." differed from *R. montetyi* in four positions in 18S rDNA. The ITS rDNA and β-tubulin gene sequence analyses provided higher resolution for comparison than 18S rDNA: "*R.* sp." and *R. quercivora* formed two distinct groups, and the nucleotide distance between them was significantly high as 7.8 % (37 out of 558 characters were different) in the ITS rDNA and 11.7 % (63 out of 881 characters) in β-tubulin gene.

Morphological analysis

The morphological characteristics of the Korean isolates ("*R.* sp.") were closest to *R. quercivora* and *R. montetyi.* This is in agreement with the phylogenetic analyses. All three species grow rapidly, form abundant aerial mycelium, and do not produce synnemata. However, they are easily distinguished by conidial size (Fig. 2); the Korean isolates produced conidia that were larger (4.8–8.3 × 2.6–3.6 μm) than *R. quercivora* (3.1–4.7 × 2.0–2.4 μm) and smaller than *R. montetyi* (6.6–13 × 3–6.6 μm). The proposed new *Raffaelea* sp. was also easily separated from *R. montetyi* by the narrow conidial width and inconspicuous annellations at the point of conidial dehiscence.

Taxonomy

Raffaelea quercus-mongolicae K.H. Kim, Y.J. Choi & H.D. Shin, **sp. nov.** Fig. 2

MycoBank MB 515072

A Raffaelea quercivora *conidia grande differt. Socius cum* Platypus koryoensis.

Holotype: **KOREA. Pocheon**; Gwangreung Experimental Forest, isolated from discolored sapwood in *Quercus mongolica* Fisch. infested by *Platypus koryoensis* (Murayama), 12 May 2005, K.H. Kim (**Holotype**: KACC44405). Sequences ex-type: GQ225700 for 18S rDNA, GQ225694 for ITS rDNA, and GQ225688 for β-tubulin gene.

Etymology: '*quercus-mongolicae*' refers to the scientific name of host plant.

Colonies on PDA maximum growth at 20–25°C, effuse, growing rapidly, reaching 90 mm diameter in 5 days with uneven white margin, appearing water-soaked and mucilaginous; colonies after 2 weeks turning brown to pale olive,

with a yeasty odor. Mycelium aerially abundant, reaching 1 cm high; hyphae branched, septate, hyaline, smooth. Conidiophores formed in sporodochia or produced separately, hyaline, straight to slightly curved, mostly aseptate, mostly single but rarely branched, smooth, variable in length as (12.5–)14.5 –*28.3*–42.2(–63) µm, but constant in width as 1.5–2.5 µm. Conidiogenous cells terminal, hyaline, smooth, proliferating sympodially or percurrently, with a series of flat, inconspicuous protruding scars or annellations. Conidia produced in acropetal order, hyaline, aseptate, smooth, thin-walled, obovoid to pyriform or oblong, (4–)4.8–*6.6*–8.3(–10) µm long, (2.2–)2.6–*3.1*–3.6(–4.0) µm wide, length/width ratio = (1.33–)1.64–*2.09*– 2.55(–3.33), tapered markedly toward the base, apex rounded, base truncate.

Habitat: Associated with *Platypus koryoensis* on living or dead stems of *Quercus mongolica*, *Q. aliena* Blume, and *Q. serrata* Thunb.

Additional isolates examined: **KOREA**, Seoul, Mt. Surak, from *Quercus mongolica*, 19 Jan 2007, K.H. Kim (KACC44404); **KOREA**, Goyang, from *Q. mongolica*, 14 Feb 2007, K.H. Kim (KACC44403).

Discussion

Raffaelea quercus-mongolicae, an ambrosia fungus closely associated with *Platypus koryoensis*, has recently contributed to significant mongolian oak mortality in Korea. The isolates were morphologically and molecularly closer to *R. quercivora* than to other *Raffaelea* species, but several distinct characteristics allowed the separation between the two species.

Conidial morphology has been commonly used in *Raffaelea* taxonomy (Scott & Toit 1970, Sutton 1975, Kubono & Ito 2002), and in this study it has also helped discriminate *R. quercus-mongolicae* from *R. quercivora*. Morphologically, the Korean isolates were easily distinguished from Japanese ones by the larger conidia. Based on 18S rDNA phylogenetic analyses, *R. quercus-mongolicae* is distinct from all other *Raffaelea* species except *R. quercivora*. The ITS rDNA and β-tubulin regions, however, show significant sequence divergences between the two species, thus further supporting an independent taxonomic position for *R. quercus-mongolicae.*

A different ambrosia beetle species is associated with each *Raffaelea* species: *Platypus koryoensis* with *R. quercus-mongolicae* and *P. quercivorus* with *R. quercivora*. This correlates with the geographic distribution of the two ambrosia beetles; *Platypus koryoensis* is commonly found in Korea but is not yet known in Japan, while *P. quercivora,* which occurs in east and southeast Asia, is not currently known in Korea. Although Gebhardt et al. (2004) suggested the possibility of ambrosia fungi switching between beetle species, many previous studies have demonstrated that an ambrosia beetle might be highly associated with only a single fungus, which appears to be the case for the species-specific

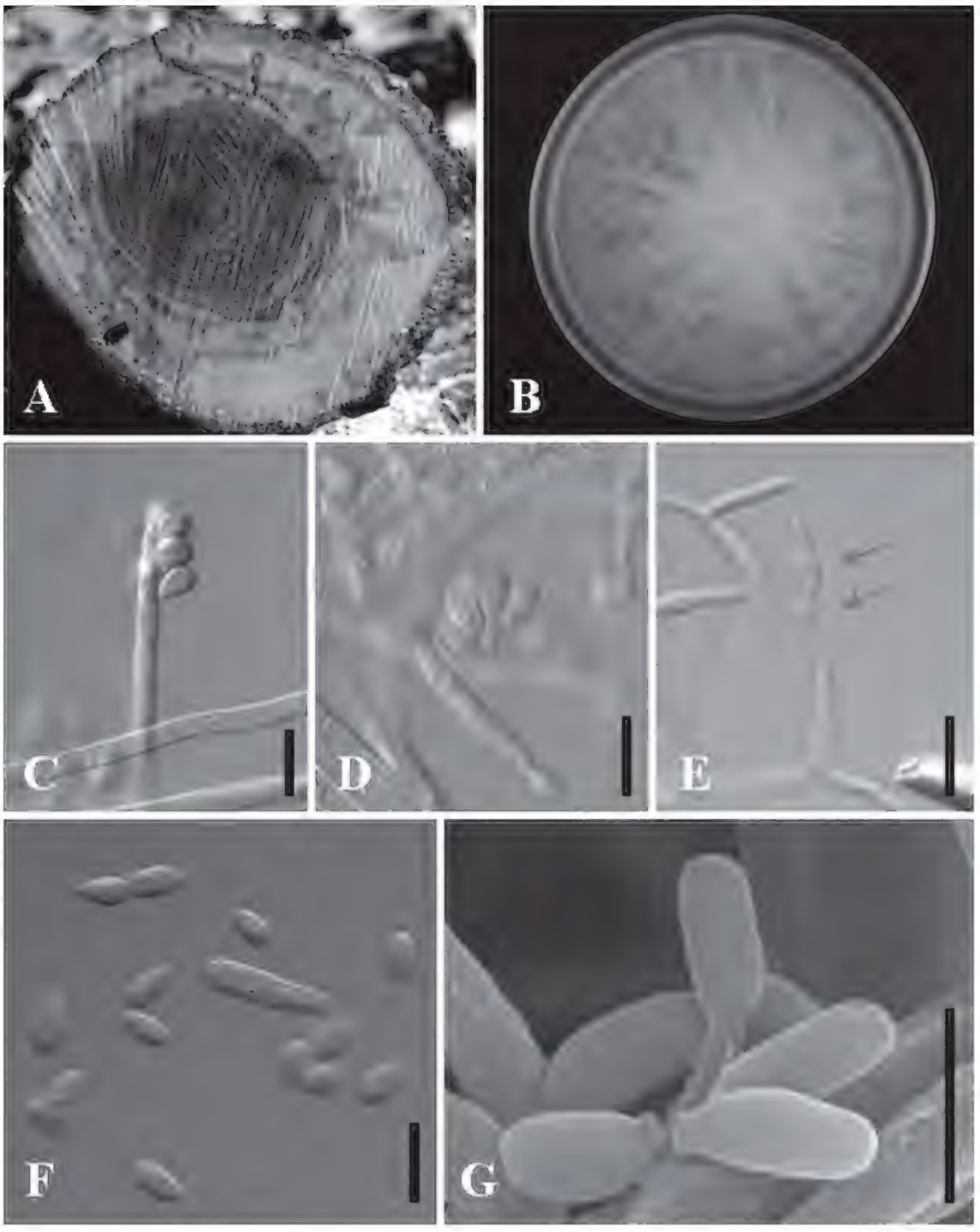

FIGURE 2. *Raffaelea quercus-mongolicae* on *Quercus mongolica*. A: Cross section of *Quercus mongolica* infested by *Platypus koryoensis*, showing necrosis (arrow); B: Colony with sporulation after 5 days of incubation at 25°C on PDA; C & D: Conidiogenous cell with sympodial proliferation and conidia; E: Conidiogenous cell with a series of flat cicatricial scars (arrow); F & G: Conidia. Scale bars = 10 µm for C-F and 5 µm for G.

association of *R. quercus-mongolicae* and *R. quercivora* with their respective ambrosia beetles.

The host plant range also differs, with the Korean species isolated mainly from *Quercus mongolica* (only rarely from *Q. aliena* and *Q. serrata*) and the Japanese species isolated from *Q. serrata* and *Q. crispula*. This coincides with the host plant geographic distribution, as *Quercus mongolica* is widely distributed over central Korea while the *R. quercivora* hosts, *Q. serrata* and *Q. crispula*, are restricted to southern Korea.

The ambrosia beetle, *P. koryoensis*, was first recorded from Korea in 1930 (Hong et al. 2006), but until now the oak mortality and its associated fungus have never been reported. The "oak death" may be closely related to recent global warming, which has allowed ambrosia beetles to extend their distribution range in Korea, as Kamata et al. (2002) noticed for Japan.

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